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Analysis of CAV VP3 Apoptin as a Potential Cancer Therapeutic

A Major Qualifying Project

Submitted to the Faculty
Of the
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In Partial Fulfillment of the Requirements for the
Degree of Bachelor of Science
By

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Approved:

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This report represents the work of one or more WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on its web site without editorial or peer review.

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Abstract

Chicken Anemia Virus Viral Protein 3 (VP3), named Apoptin, selectively induces G2/M arrest and apoptosis in human cancer cells without affecting normal primary cells. Apoptin engages in nucleocytoplasmic shuttling in cancer cells and normal primary cells; post arrest it is observed that in infected cancer cells Apoptin localizes to the nucleus, whereas Apoptin is observed in the cytoplasm of normal primary cells. The N-terminal Nuclear Export Sequence of Apoptin is suspected to also contain a domain responsible for protein multimerization activity. For our investigation we have attempted to uncouple multimerization from nucleocytoplasmic shuttling to determine if both are required to induce apoptosis in cancer cells. We have determined that elimination of nuclear export and diminished multimerization capacity of Apoptin is sufficient to induce apoptosis. Further research into the connection between nuclear export and multimerization will need to be conducted in order to determine if synthesis of a small molecule therapeutic will be able to duplicate the functionality of Apoptin.

Introduction

Chicken Infectious Anemia

The Chicken Anemia Virus (CAV) is a *Gyrovirus* of the *Circoviridae* family that causes complete multi-system failure in infected chicken subjects and eventually death (1). The virus was first isolated in Japan, with the resulting condition referred to as Blue Wing Disease or Chicken Infectious Anemia. CAV is highly contagious and is predominantly seen in large flocks of chickens raised for human consumption called broilers (8). The virus is capable of both horizontal and vertical transmission. CAV can be passed vertically from a hen to her offspring in utero; offspring infected with CAV exhibit symptoms of infection upon hatching, which include but are not limited to pale color, lethargy, and bluish discoloration of the wing. Anemia is often confirmed through blood testing (1). CAV can also be transmitted from rooster to hen and vice versa during sexual reproduction, although the primary path of infection has been confirmed to be respiratory inhalation of virions found in chicken excretions. This correlates with the high instance of CAV found in larger flocks of broilers kept in close proximity to one another.

CAV is considered to be a “wasting virus” due to its effect on multiple systems of the infected subject. The virus induces significant anemia in the subject, causing lethargy (1). The virus also suppresses the number of T-cells produced by the lymphocytes of the host, causing weakened immunity (1). Decreased immunity in the subject typically leads to parallel infection by other pathogens affecting critical systems of the body. Most commonly these parallel infections are determined to be the cause of death.

Immunosuppression of infected subjects is caused by high concentration of virions in the cortex of the thymus as well as Cluster of Differentiation 8 (CD8) cells of the spleen (1). Virion activity in these locations inhibits maturation of killer T cells required to generate typical

immune responses. CAV is also found in high concentrations in the hemocytoblasts of the bone marrow of infected subjects. Infection of the hemocytoblasts is hypothesized to be the primary cause of chronic anemia in infected subjects. Bone marrow of infected subjects is observed as yellow to pale yellow in color; blood smear tests conclude that clotting times are longer than those of healthy subjects and that the blood of infected subjects has a “watery” consistency (1). Confirmation of CAV infection in chickens is attained by identification of viral DNA in the thymus or in the bone marrow. Dissection post mortem shows severe atrophy of the thymus of infected subjects. Hemorrhaging may also be present in excess.

Details of CAV Structure

CAV measures a total of 25 nanometers in length and contains a single stranded circular genome that codes for three viral proteins (Figure 1); the genome is estimated to be around 2300 base pairs (8). Viral protein VP1 is the capsid protein of the virus, whereas VP2 is hypothesized to be a scaffolding protein required for correct folding of VP1. The third viral protein coded for by CAV is VP3 which is called Apoptin; the function of Apoptin is to initiate cell death in infected cells (4).

Sequence and Structure of Apoptin

In order to better understand Apoptin and its function, its sequence and structure must be identified. Apoptin is comprised of 121 amino acids and is rich in serine (Ser) and threonine (Thr) residues. Apoptin has a Nuclear Localization Sequence (NLS) at its C-terminal end, as well as a Nuclear Export Sequence (NES) at its N-terminal end. Previous studies have determined that the NLS is composed of amino acids 70-121 while the NES is assumed to consist of amino acids 37-46 (4); the NES and NLS are necessary for nucleocytoplasmic shuttling. Nucleocytoplasmic shuttling gives Apoptin its ability to shuttle between the nucleus and the cytoplasm in both

primary and transformed cells. In primary cells, Apoptin is partitioned in the nucleus, as opposed to the cytoplasm, which is seen in transformed cells (2). Amino acids 33-46 show a high concentration of hydrophobic leucine. This significant concentration of hydrophobic leucine is critical for self-association. Exogenously expressed Apoptin can form a non-covalent multimeric complex that consists of 30-40 monomers; each one of these monomers is comprised of a hydrophobic proline at its N-terminal.

CAV Functionality in Transformed Cells

The VP3 protein Apoptin synthesized by CAV has a unique property when exposed to human tissue cultures. Previous research has concluded that transformed (cancerous) cells infected with a vector containing the gene for expressing VP3 (Apoptin) synthesis undergo apoptosis. Some of these transformed cells include tumor cells such as melanoma, hepatoma, lymphoma, cholangiocarcinoma, colon carcinoma, breast cancer, and lung cancer cells. Apoptin also causes apoptosis in SV40-transformed fibroblasts or UV-irradiated cells from individuals with hereditary cancer-prone syndromes (5). Examination of infected cell cultures determined that a high concentration of Apoptin was present in the nucleus of each transformed cell (4). Alternatively, when the gene for Apoptin synthesis was expressed in infected normal cells, cell death was not observed. Human endothelial cells, hepatocytes and hematopoietic stem cells are examples of normal cells that are not susceptible to Apoptin. Examination of infected normal cells determined that Apoptin was found in the cytoplasm. It is therefore hypothesized that a mechanism for the selective killing of transformed cells by Apoptin must be present (4).

The functionality of Apoptin in transformed cells was originally discovered in the late 1970's while studies were conducted initially on CAV. The purpose of these initial studies was to understand the nature of the virus that was wiping out entire flocks of chickens. An

unintentional discovery was made when scientists observed that the virus was inducing arrest in transformed cell lines that were mutated from an initial normal cell line (4). Further research on the killing effect of CAV *in vivo* was performed where it was determined that the virus was inducing cell death by means of VP3 Apoptin. When spliced into a vector capable of infecting human tissue, it was observed that Apoptin induced cell death with a high level of efficiency in carcinoma cells whereas having no effect on normal cells. This determination gave rise to the current study of CAV and the potential application of Apoptin as an anti-cancer treatment option.

It is known that Apoptin is a nucleocytoplasmic shuttling protein that is localized based on the N-terminal nuclear export sequence (NES) and the C-terminal nuclear localization signal (NLS). It is hypothesized that Apoptin functions by shuttling from the cytoplasm of a cell to its nucleus and back into the cytoplasm. This cycle is thought to be continuous. It is unknown why Apoptin localizes in the nucleus of transformed cells and in the cytoplasm of normal cells. It is hypothesized that the NES complex of Apoptin is responsible for both protein export and multimerization.

The Anaphase Promoting Complex and p53 Anti-tumor Pathway

Currently it is believed that nuclear export and multimerization capacity are what give Apoptin its ability to limit the activities of the Anaphase Promoting Complex/Cyclosome (APC/C) to promote apoptosis. This activity occurs independent of the p53 pathway to induce apoptosis, a pathway which has evolved in eukaryotic metazoans as a master regulator to protect the organism from cancer proliferation (4). The p53 protein is an anti-tumor agent that oversees cellular activity and is capable of inducing arrest if cancerous activity is present (10). The primary function of p53 is to promote expression of a wide array of genes to try to correct the errors occurring in the cell. Prolonged activation of the p53 anti-tumor pathway will result in

apoptosis. It is approximated that in excess of 50% of all cancers have ineffective p53 anti-tumor activities. The protein Apoptin interacts directly with the APC/C to induce apoptosis independent of this pathway.

The APC/C is utilized to regulate phase transition in the cell cycle. One activity of the APC/C that is of interest to this project is that the APC/C is utilized by the cell to transition itself from metaphase to anaphase by forcing the separation of sister chromatids. The complex itself consists of 13 subunits wrapped around a central protein called Apc1. In terms of this project's research, Apoptin interacts directly with the central Apc1 protein of the APC/C to prevent the cell from progressing into metaphase and induces G2/M arrest (4).

VP3 Apoptin Inhibits APC/C Activity

It is imperative to note that inhibition of APC/C activity by VP3 Apoptin during the separation of sister chromatids in metaphase is the source of arrest induction. Although the precise mechanism of action between Apoptin and APC in cancer cells is unclear, it is known that this interaction is necessary for Apoptin to induce specific G2/M arrest (4). All eukaryotic cells undergoing cellular mitosis enter 4 different stages during the cell cycle; although the APC/C plays important roles in all phases of the cell cycle, only the G2/M phase is of importance to the study of Apoptin. The 4 phases of the cell cycle occur sequentially as G1, S, G2, and M. Immediately following the S phase that a very brief gap phase termed the G2 phase occurs: it is at the end of this gap phase that a control checkpoint takes place to determine if the cell can pass on to the final phase or if cell division needs to be aborted (9). After passing this checkpoint, the cell enters the final phase of the cell cycle called the M phase. The M phase is phase in which cellular mitosis takes place, and the cell divides into two daughter cells. G2/M arrest induction by VP3 Apoptin occurs as the name would imply directly between the transition

from the G2 gap phase to the M phase, where interaction with the APC/C locks the cell from phase progression. It is believed that extensive arrest experienced during the G2/M phase is what will ultimately commit the cell to apoptosis.

Induction of Point Mutations

In order to better understand the functionality of Apoptin and its interaction with the APC/C, it is vital to attempt to uncouple the overlapping activities of nuclear export and multimerization. Figure 2 shows the amino acid sequence for wild type Apoptin; the sequence stretch from isoleucine 33 to leucine 46 is of particular interest to this project because it contains the overlapping domains of VP3 Apoptin's nuclear export and multimerization. Based on previous research conducted by Heilman et al, point mutations were induced in the amino acid sequence of the protein Apoptin. Heilman's research concluded that mutating key amino acids of Apoptin's NES to alanine would knock-out nuclear export. The initial mutant pm-NES replaced leucine 44 and leucine 46 with alanine, rendering nuclear export inactive but also diminishing multimerization capacity of the protein by approximately 50%. Individual substitutions for alanine were then made at isoleucine 40, threonine 43, leucine 44, and leucine 46 to attempt to eliminate nuclear export without affecting the multimerization capacity of the protein.

This relates to the current study of Apoptin in two ways. First, it was observed by Tischer and Brasefield after performing a yeast 2 hybrid assay that the results were ambiguous. One possible reason for those results is based on the components of the assay. Brasefield and Tischer attempt to cross a wild type Apoptin gene inserted into the pACT2 vector with their 4 mutants that had been inserted into the pGBKT7 vector. To provide a better indication of the effects of multimerization in these mutants, this study is attempting to repeat the yeast 2 hybrid assay replacing the wild type Apoptin pACT2 vector with the mutated copy for the gene inserted into

the pACT2 vector. The second aspect of this study is to assess the killing capacity of the pm-NES mutant. The purpose of this study will be to determine how a mutant completely lacking nuclear export capability and diminished multimerization capacity will behave when introduced into human cancer cells.

Materials and Methods

Molecular Cloning

Restriction digests were set up by preparing 4 samples consisting of 15 μL of sterile ddH₂O, 2 μL of 10X Buffer E, 1 μL of NCO1, 1 μL of Bam HI, and 1 μL of insert, with mutants I40A, L44A, L46A, and T43A. The samples were then placed in a 37°C water bath for 1-3 hours. The gene of interest from each sample was then isolated utilizing a 0.9% agarose gel electrophoresis. The gel was assembled using 1X TAE (Tris base, acetic acid, and EDTA) and ethidium bromide. The genes of interest were purified in accordance with the Geneclean purification kit found elsewhere (7).

The genes of interest were then inserted into the pACT2 vector via ligation reactions. Two ligation reactions were set up for each mutant insert. The 1:1 reaction consisted of combining 2 μL of pACT2, 2 μL of insert, 2 μL of Buffer E, 13 μL of water, and 1 μL of ligase. The 1:3 reaction consisted of combining 2 μL of pACT2, 6 μL of insert, 2 μL of Buffer E, 9 μL of water, and 1 μL of ligase. These samples were then incubated for 1 hour at room temperature. Post incubation, the 4 sample vectors were transformed into a volume of 50 μL of *E. coli*.

A total of 10 μL of each sample was transferred to each appropriate tube of *E. coli*. Samples incubated on ice for 20 minutes. Samples were then heat shocked in a 42° Celsius water bath for exactly 60 seconds. The samples were then transferred back to the ice for 2 minutes. A volume of 500 μL of LB media was added to each sample. A volume of 200 μL of each sample was plated onto the appropriate ampicillin plates. The plates were then incubated at 37° Celsius overnight.

The next day, the colonies were inoculated. The amount of total LB media needed was calculated by multiplying 3 mL by the number of colonies inoculated. A volume of 1000x

ampicillin was added to the media. A number of 3 mL aliquots of media was then prepared. Each colony of interest was isolated and added to media. Samples were then incubated for 24 hours.

Purification of Plasmid DNA from E. coli

The plasmid DNA was purified from E. coli. 1.5 milliliters of each culture was transferred and centrifuged at 3000 x g for 5 minutes; the supernatant was decanted. Another 1.5 mL of each sample was transferred and centrifuged at 2000 x g for 5 minutes. The supernatant was decanted and the bacterial pellets were re-suspended in 100 µL of MPS I solution. A volume of 200 µL of MPS II solution was added to each sample. The samples were incubated at room temperature for 3 minutes. Then, 150 µL of MPS III solution was added to each sample. The cells were placed on ice for 10 minutes, followed by a 5 minute centrifugation process at 12,000 x g. The supernatant was removed and two volumes of cold 100% ethanol were added to each sample. The cells were placed on ice for 2 minutes, followed by a 5 minute centrifugation at 12,000 x g; the supernatant was decanted and 1 mL of 70% ethanol was added to rinse the pellets. The samples were centrifuged at 12,000 x g for two minutes. The ethanol was removed and the pellets were re-suspended in 50 µL 1 x Tris EDTA (TE).

Apoptosis

A series of apoptosis assays were performed using the Promega Apo-One Homogeneous Caspase-3/7 Assay Kit and protocol found elsewhere (6). Utilizing H1299 non-small cell lung carcinoma cells, killing capacity of pm-NES was analyzed. The assay induces significant quantifiable fluorescence in cells that undergo apoptosis by means of caspase-3 activity. H1299 cells were cultured in DMEM with 10% Fetal Bovine Serum (FBS) and 1x PSF (Penicillin Streptomycin Fungicide) and passed into a 96 well plat with 1 milliliter of media 1 day before transfection to achieve approximately 70% confluence. Transfection with pm-NES was

performed using Effectene; positive and negative controls were set up in groups of three independent wells, with transfection occurring in a cluster of three wells. After 1 day of incubation, the media from each well was aspirated and replaced with 50 μ L of fresh media and 50 μ L of caspase-3/7 buffer. Results can then be recorded after an 18 hour incubation period by means of fluorometry.

Results

Vector Transformation

Removal of the 4 mutants prepared by Brasfield and Tischer from the pGBKT7 vector was attempted in order to successfully insert them into the pACT2 vector for the purpose of performing a yeast 2 hybrid assay. Excision of the 4 mutated genes for CAV VP3 Apoptin was successful (Figure 4). The ladder conformation of the marker used in lane 1 serves to visualize approximate fragment sizes of samples. Smaller molecules are able to travel further down the gel during electrophoresis due to smaller sieving interactions and greater charge interactions. The smearing effect seen in lane 2 is the result of purified pGBKT7 vector run on the assay without implementation of restriction enzymes. The visible banding is the result of linearized versus compact DNA; compact DNA is capable to migrate farther down the lane than DNA that has unfolded into the plasmid conformation which must overcome greater sieving forces. Lanes 3 and 4 each show the pGBKT7 vector cut with BamH1 and Nco1 respectively. These bands run at approximately 2.3 Kb as the plasmid is now linearized. Lane 5 shows the vector running at approximately 2 Kb with the mutated Apoptin gene excised and running at approximately 0.3 Kb. Ligation of these inserts into the pACT2 vector is currently in progress. E. coli transfections using ampicillin resistant selection can then be achieved.

Apoptosis Killing Experiments

While tackling the problem of uncoupling nuclear export and multimerization, killing capacity for the best mutant available, pm-NES, was assessed. Although this mutant had zero nuclear export activity, roughly 50% of its ability to multimerize had been eliminated. Nonetheless, evaluating the ability for this mutant to induce apoptosis resulted in startling conclusions. The results of the Apo-One assay utilizing H1299 cells and pm-NES show that

eliminating Apoptin's ability for nuclear export and diminishing its multimerization capacity are sufficient to induce apoptosis (Figure 3). The bottom curves of the figure show fluorescence data at 510 nm for the negative controls of the assay where H1299 cells were not transfected with DNA but were carried through the entire experiment and subjected to the same reagents. The upper curves of the graph are fluorescence data recorded for the positive controls of the assay which were treated with native Apoptin. These positive controls exist to show what apoptosis mediate by caspase-3 should look like. The labeled curves in the figure are the results of the assay which had collected fluorescence data within the bounds of the controls. 1 of the mutant assay wells had minimal apoptotic activity on the same level as the negative controls. The remaining two mutant assay wells for the experiment yielded robust apoptotic activity on the same level as the positive controls for the assay.

Discussion

Localization and Multimerization

The study of CAV VP3 Apoptin has been in progress for several years. It is known that Apoptin is capable of inducing G2/M arrest and apoptosis (4). Several studies over the years have indicated that localization and multimerization are two important components in Apoptin's ability to induce apoptosis. Understanding the affects that localization and multimerization have on Apoptin can be used to ones advantage; one can better understand Apoptin's capabilities and functions by performing new experiments based on what has already been declared.

Regarding Apoptin's localization, it has been determined that Apoptin is localized in the nucleus of transformed cells, as opposed to the cytoplasm, where Apoptin is localized in non-transformed primary cells (4). Apoptin's nuclear export sequence is believed to play a role in its localization due to the fact that the specific Apoptin NES is required for cell type-specific localization (4). Regarding multimerization, Green, Heilman, and Teodoro have suggested that Apoptin's multimerization domain overlaps with its nuclear export sequence. However, Apoptin's multimerization is unique in the fact that it appears to vary in degree between cell types and affects Apoptin's localization and apoptotic activities (4). Primary cells have higher conditions of multimerization. This is a possible explanation as to why Apoptin has aggregation, insolubility, and retention characteristics in the cytoplasm (4). Experiments have shown that multimerization is required to establish a high-affinity interaction with APC1. Green, Heilman, and Teodoro have shown that there was a reduced affinity for APC1 when the Apoptin mutants exhibited decreased multimerization capacity. Comprehending the role multimerization plays in Apoptin is vital to understanding its biochemical behavior.

Comparison to Previous Studies

Apoptin's localization and multimerization connections that were derived from previous studies provided a basis for the attempts to determine whether or not multimerization and nuclear export can be uncoupled. Based on the pm-NES mutant, apoptosis induction was observed with diminished multimerization and lack of nuclear export; this indicates that nuclear export is not necessary to induce apoptosis. On the other hand, minimal multimerization is sufficient to induce apoptosis. Since the induction of apoptosis was observed, there must have been an interaction with APC1, which lead to G2/M arrest. This agrees with previous studies that concluded that multimerization is required to establish a high-affinity interaction with APC1. Based on previous conclusions, it was expected that there might be a reduced affinity for APC1 due to minimal multimerization. However, a reduced affinity for APC1 was not a problem in this situation. An explanation to this, as mentioned earlier is because multimerization appears to vary in degree between cell types, which affects apoptotic activities. So, the cells that were used in this specific experiment could have affected the results. Since transformed cells have lower conditions of multimerization, Apoptin has less aggregation, as well as insolubility in the nucleus, which accounts for its ability to induce apoptosis without nuclear export.

Overlapping Domains

Previously it has been shown that the nuclear export sequence and multimerization domain overlap on the amino acid sequence. What is not known about this overlap is how it is functionally possible. It is hypothesized that although the domains are overlapping, the active sites that these amino acids create exist on opposing sides of the same subunit in 3-dimensional space. On one side, the nuclear export sequence exists to promote export activity; opposite can be found the active site responsible for protein multimerization. This theory presented by

Heilman is based on the idea that it is highly unlikely that the overlapping domains physically share the same active site in the 3-dimensional space. It is possible then that any mutation made to this sequence will alter the conformation of the subunit and make it impossible to create a mutant that completely lacks on activity while entirely maintaining the other.

Yeast 2 Hybrid Assay

One of the most useful tools in testing multimerization capacity is the usage of the yeast 2 hybrid assay. In evaluating the multimerization capacity of future mutants, this assay is efficient in producing visual, qualitative results. Using YBD plates negative for leucine and tryptophan, selective proliferation of yeast will occur. Utilizing Y190 cells, a cross of two vectors, each containing the same mutant VP3 gene, will be transformed into the cell. Each of these vectors contains a cassette allowing for proliferation of colonies in a negative leucine and negative tryptophan environment. Cells that have successfully been transformed by these vectors will then be able to colonize on these selective plates. If the transformed vectors are able to multimerize, the promoter for the LacZ operon will be activated (Figure 5). Using X Gal and β -mercaptoethanol, colonies with active multimerization will be stained blue. This qualitative data can be used to assess the multimerization capacity of the mutant of interest (3).

Future Apoptosis Experiments

Although the results of the apoptosis experiments using the Apo-One kit reveal provocative information, there is still significantly more data that can be collected using the pm-NES mutant. Whereas there is little doubt now that decreased multimerization activity and non-functional export is sufficient to induce G2/M arrest and apoptosis in cancer cells, there is no information to support that Apoptin's selectivity has remained intact. It is proposed that the same

apoptosis experiment be repeated, except with non-transformed primary cells instead of H1299 non-small cell lung carcinoma cells.

The reason why Apoptin has been of great interest is its ability to selectively target and kill human cancer cells without effecting normal cells. It is unknown if eliminating nuclear export has an effect on this selectivity. There is no current information to support a hypothesis that elimination of nuclear export will have no effect on the selectivity of Apoptin's killing capacity; there is also no evidence to not support this theory. In short, an apoptosis assay for the killing capacity of the pm-NES mutant in non-transformed primary cells is necessary. If the results of this assay indicate that Apoptin's selectivity has been eliminated, then it can be determined that the selectivity of VP3 Apoptin is linked to the activities of nuclear export and multimerization. Continuing the attempt to uncouple these activities is also of significant importance because a similar test will need to be performed in which full multimerization capacity is retained.

Apoptin Selectivity and Small Molecule Therapeutics

The ultimate goal in studying the biochemistry of VP3 Apoptin is to one day learn enough about the protein's behavior and attempt to synthesize a small molecule therapeutic that can recapitulate that behavior. Results indicate that nuclear export and diminished multimerization are sufficient to induce apoptosis in H1299 cells, but whether or not these activities play a role in Apoptin's selectivity is still unknown. If on one hand it is discovered that nuclear export is necessary to induce selective apoptosis in H1299 cells, then the idea of creating a small molecule therapeutic will be essentially impossible. The complex interactions necessary to cross the specific nuclear envelope and perform an additional function are currently far beyond scientists' abilities to replicate with a small molecule; this type of action can only be

achieved with a protein. However, if multimerization and protein solubility are what drives Apoptin's selectivity, as this project's results appear to indicate, then the concept of a small molecule therapeutic still has potential.

Nuclear Export Manipulation

Another compelling question is how Apoptin would behave if it were to contain a domain for functionally inactive nuclear export as well as an active domain for nuclear export. It is possible that such a mutation will have no effect, and it is possible that by introducing a secondary nuclear export sequence will have a bolstering affect on inducing apoptosis. One possibility is to utilize the nuclear export sequence from another protein and splice it into VP3 Apoptin. These types of experiments will be able to shed some light on the functionality of Apoptin by simply yielding positive or negative results. It is possible that having two functioning nuclear export sequences both maintaining complete multimerization capacity will accelerate the arrest process. Manipulation of the nuclear export sequence in manners such as these will help shed light on its purpose.

Multimerization Manipulation

Conversely of the nuclear export sequence, it has been demonstrated that Apoptin with approximately 50% of its multimerization capacity is still capable of inducing G2/M arrest. These results indicate that multimerization plays some significant role in Apoptin's functionality. Useful future experiments into the manipulation of multimerization are to test the protein's killing capacity without nuclear export and with multimerization at full strength and also at a minimal strength. It is possible that by decreasing Apoptin's ability to multimerize to a greater extent will allow it to remain more soluble within the cell, an observation characteristic of Apoptin in the nucleus of transformed cells. It is also entirely possible that further limiting the

multimerization capacity of Apoptin will completely eliminate its ability to induce apoptosis.

Regardless of the result, altering the multimerization capacity of Apoptin will yield results

indicating that the extent to which the protein can multimerize may affect the protein's ability to induce G2/M arrest.

Figures

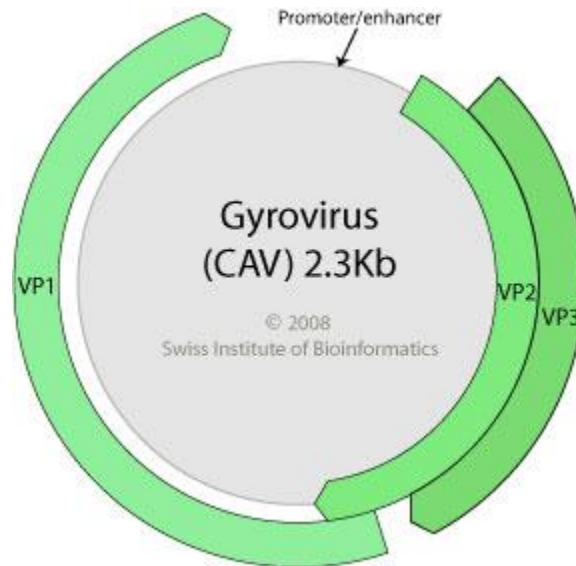


Figure 1: This map of the Chicken Anemia Virus shows the domain for each of the three proteins synthesized by the virus. The protein of interest for this study was VP3, called Apoptin, which shares an overlapping domain with VP2 which is believed to be the scaffolding protein of the virus. The entire CAV genome is 2300 base pairs long (2.3 Kb).

1	11	21	31	A
MNALQEDTPP	GPSTVFRPPT	SSRPLETPHC	<u>REIRIGIAGI</u>	
41	51	61	71	
AA A	NARAPTLRSA	TADNSESTGF	KNVPDLRTDQ	
<u>TITLSLCGCA</u>				
81	91	101	111	121
<u>PKPPSKKRSC</u>	DPSEYRVSEL	KESLITTTPS	<u>RPRTAKRRIR</u>	L
NLS1			NLS2	

Figure 2: This sequence map of VP3 Apoptin shows in red lettering where point mutations were induced in an attempt to uncouple multimerization and nuclear export. Apoptosis experiments using the pm-NES mutant with a double mutation at leucine 44 and leucine 46 for alanine determined that nuclear export had been eliminated.

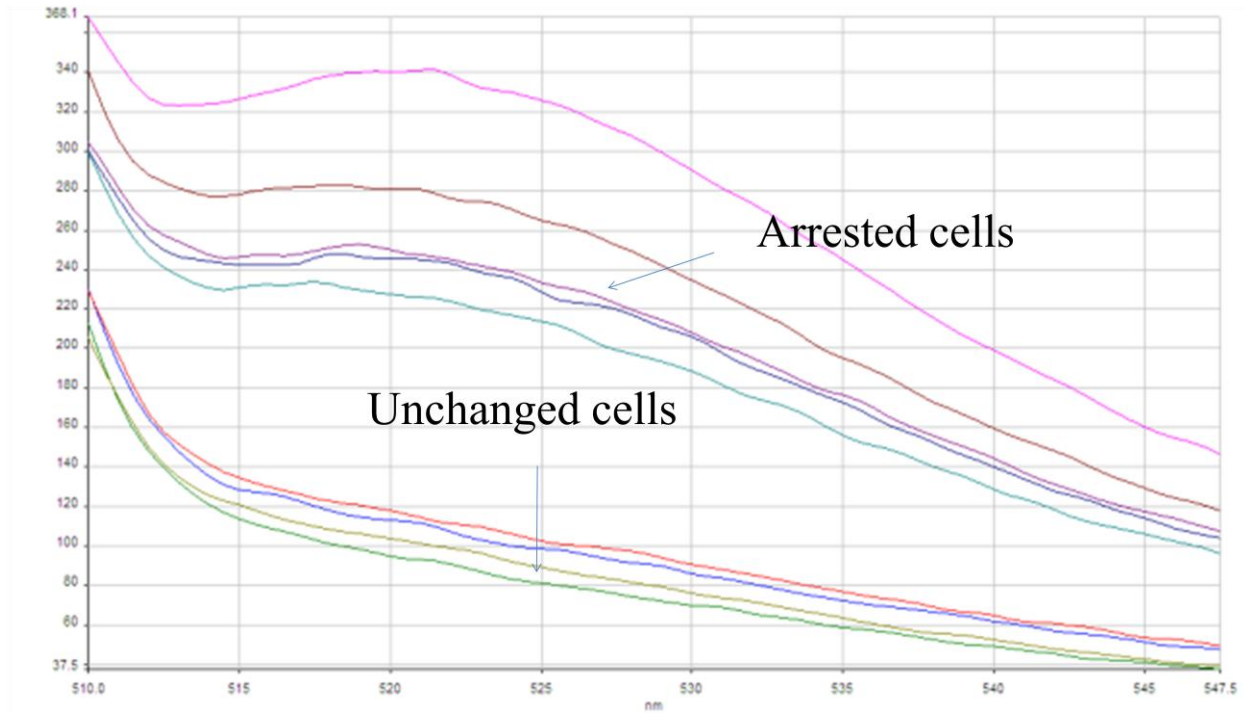


Figure 3: A chart of fluorescence data results from pm-NES apoptosis killing experiment trial 2 with H1299 cells. This chart shows that of the three wells tested, 2 wells had significant apoptosis activity (purple and blue curves). The third well in this assay had minimal apoptotic activity within the bounds of the negative controls.

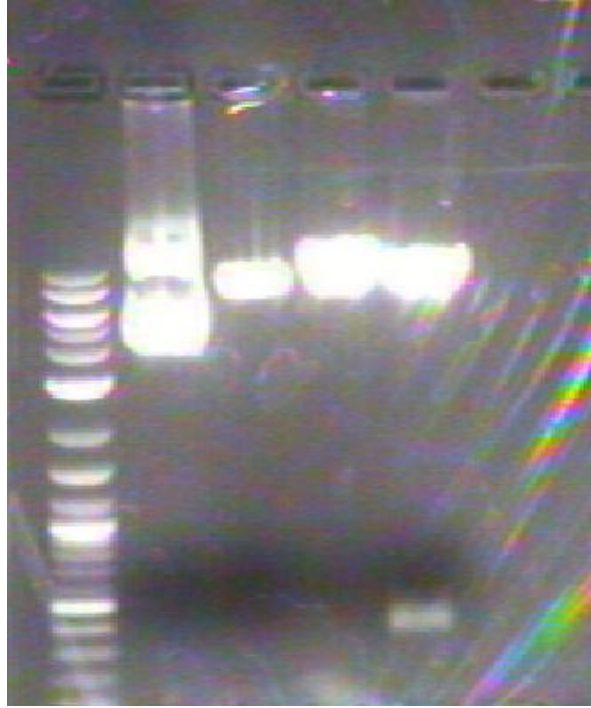


Figure 4: This image is of a 0.9% agarose gel used to assay via electrophoresis the L44A mutant ligated into the pGBKT7 vector. Lane 1 was loaded with Marker; lane 2 was loaded with 2 μ L of purified L44A mutant; lane 3 was loaded with L44A mutant cut with BamH1 restriction enzyme; lane 4 was loaded with L44A mutant cut with Nco1 restriction enzyme; lane 5 was loaded with L44A mutant cut with BamH1 and Nco1. The image shows a band drop out in lane 5 which is representative of the mutated Apoptin insert.

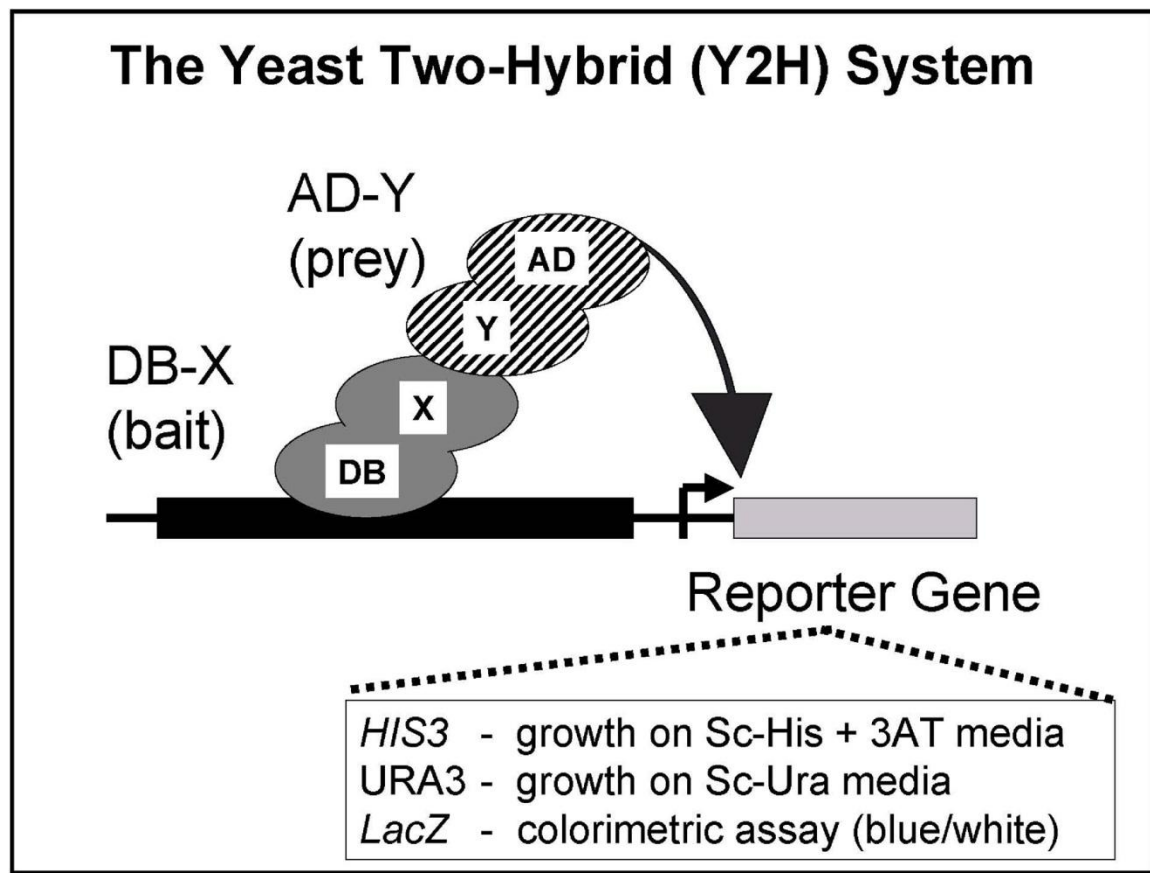


Figure 5: A schematic for the operation of the Yeast 2 Hybrid system in which two vectors each containing one half of the promoter for the reporter gene come together in order to activate the operon (Walhout). For this project, the *LacZ* operon was used as the reporter gene to test for the multimerization capacity of mutant Apoptin genes. In the presence of X Gal and β -mercaptoethanol, *LacZ* positive yeast colonies will turn blue.

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